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Associations Between ADH Gene Variants and Alcohol Phenotypes in Dutch Adults

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Recently, Macgregor et al. (2009) demonstrated significant associations of ADH polymorphisms with reactions to alcohol and alcohol consumption measures in an Australian sample. The aim of the present study was to replicate these findings in a Dutch sample. Survey data on alcohol phenotypes came from 1,754 unrelated individuals registered with the Netherlands Twin Register. SNPs in the ADH gene cluster located on chromosome 4q (n = 491) were subdivided in seven gene sets: ADH5, ADH4, ADH6, ADH1A, ADH1B, ADH1C and ADH7. Within these sets associations of SNPs with alcohol consumption measures, age at onset variables, reactions to alcohol and problem drinking liability were examined. Of the original 38 SNPs studied by Macgregor et al. (2009), six SNPs were not available in our dataset, because one of them had a minor allele frequency < .01 (rs1229984) and five could not be imputed. The remaining SNP associations with alcohol phenotypes as identified by Macgregor et al. (2009) were not replicated in the Dutch sample, after correcting for multiple genotype and phenotype testing. Significant associations were found however, for reactions to alcohol with a SNP in ADH5 (rs6827292, p = .001) and a SNP just upstream of ADH5 (rs6819724, p = .0007) that is in strong LD with rs6827292. Furthermore, an association between age at onset of regular alcohol use and a SNP just upstream of ADH7 (rs2654849, p = .003) was observed. No significant associations were found for alcohol consumption and problem drinking liability. Although these findings do not replicate the earlier findings at the SNP level, the results confirm the role of the ADH gene cluster in alcohol phenotypes.

Keywords: alcohol dehydrogenase, alcohol initiation, physical reactions, multiple testing, genetic association

Alcohol is commonly used in Western societies: the vast majority of adults report to have drunk alcohol in the previous 12 months (Ahlstrom et al., 2001; CBS, 2008; Wilsnack et al., 2000; van Laar et al., 2008). There is substantial individual variation in the amounts of alcohol consumed. Based on data from the World Health Organization 15.7% of individuals aged 15 and older are classified as heavy drinkers (males > 40g and females > 20g pure alcohol per day; Rehm et al., 2006).

The harmful effects of alcohol on health are well-documented. Increased alcohol use has been linked to over 60 chronic health diseases, ranging from cancers and heart disease to depressive disorders and birth defects (Rehm et al., 2003). Alcohol use disorder is in the top 10 conditions with the highest burden of disease (Begg et al., 2007; Hilderink & van't Land, 2008; World Health Organization, 2008). In addition, alcohol takes its toll through motor vehicle accidents and other alcohol related injuries (Rehm et al., 2003; van Laar et al., 2008). In Western European countries, 6.6% of deaths among males can be attributed to alcohol consumption (Rehm et al., 2006). However, there is also evidence that alcohol can have beneficial effects on health. For example, moderate alcohol use has been associated with a decreased risk of both coronary heart disease and ischaemic stroke (Burger et al., 2004). Given the large impact of alcohol consumption, it is important to understand the causes of individual differences in alcohol use.

Twin studies have shown that individual differences in a range of different alcohol phenotypes are all explained to some degree by differences in genetic makeup (Dick et al., 2009). Additive genetic influences explain between 30% and 54% of the variation in alcohol consumption among adults (Hansell et al., 2008; Kendler et al., 2008; Whitfield et al., 2004). Heritability estimates for the risk of alcoholism or abuse in adults range from 30% to 75% (Heath et al., 1997; Jang et al., 2000; Kendler et al., 1997; Knopik et al., 2004; Prescott et al., 1999; Sartor et al., 2009; Walters, 2002; Xian et al., 2008). Estimates of genetic influences on alcohol initiation are more variable. Although the average heritability is estimated to be around 26% (Dick et al., 2009), estimates range from 0 to 83% (Fowler et al., 2007; Han et al., 1999; Koopmans & Boomsma, 1996; Pagan et al., 2006;

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Penninkilampi-Kerola et al., 2005; Poelen et al., 2008; Rhee et al., 2003; Rose et al., 2001; Stallings et al., 1999; Sartor et al., 2009; Viken et al., 1999). The heritability of age at onset of regular alcohol use has been estimated at 35–43% (Liu et al., 2004; Stallings et al., 1999).

One of the mediating mechanisms by which genes may influence alcohol use, is through alcohol metabolism (Higuchi et al., 2006). Two groups of genes that have received much attention are the alcohol dehydrogenase (ADH) and the aldehyde dehydrogenase (ALDH) gene clusters that code, respectively, for the ADH and ALDH enzymes that are involved in the break-down of ethanol. ADH is one of the main enzymatic pathways metabolizing ethanol into acetaldehyde, a highly toxic by-product. ALDH oxidizes acetaldehyde into a less toxic compound, acetate, which is subsequently converted into water and carbon dioxide. Variants of ADH and ALDH genes encode enzymes with different characteristics that can change the rate by which ethanol is metabolized and hence influence alcohol use and other alcohol-related phenotypes (Edenberg, 2007).

ADH enzymes are encoded by seven different genes, located in a 365 kb region on chromosome 4q, that have been categorized into five classes: (a) ADH1A, ADH1B and ADH1C that encode the enzymes α-, β-, and γ-ADH; (b) ADH4 that encodes μ-ADH, (c) ADH5 that codes for χ-ADH, (d) ADH6 that encodes an ADH enzyme little is known about and (e) ADH7 that encodes σ-ADH. ALDH is encoded by two genes. Gene ALDH1A, located on chromosome 9q21.13, codes for the enzyme ALDH1. The ALDH2 gene located on chromosome 12q24.2, codes for the enzyme ALDH2. Most variants in the ADH and ALDH gene clusters are changes in single nucleotide polymorphisms, also called SNPs (Edenberg, 2007).

Macgregor et al. (2009) investigated whether genetic variants in the ADH and ALDH2 genes were associated with alcohol consumption, physical reactions to alcohol and alcohol dependence in an Australian population sample from Western European descent (n = 4,597; age range 26–89). They examined 38 polymorphisms in the ADH gene cluster and 8 polymorphisms in the ALDH2 gene. Significant associations were found between variants in the ALDH2 gene and measures of alcohol dependency. Variants in the ADH genes were found to be related to alcohol consumption measures and physical reactions and only marginally with alcohol dependency measures. The most significant finding was obtained for the association of marker rs1229984 (ADH1B) with alcohol consumption variables ($p = 2.7 \times 10^{-06} - 8.9 \times 10^{-08}$) and physical reactions ($p = 8.2 \times 10^{-07}$), but significant associations for other SNPs were also reported.

In this article we sought to replicate Macgregor et al.'s (2009) findings on the association of ADH polymorphisms with alcohol consumption measures, physical reactions to alcohol and alcohol dependency. To this end, the data from 1,754 unrelated individuals registered with the Netherlands Twin Register (NTR) were analyzed. The participants were unselected for alcohol use and comparable to the Australian sample in age and phenotypic assessments. A problem drinking scale was used as a proxy for alcohol dependency. In addition, we examined the associations between ADH polymorphisms and age at onset of alcohol use. The participants were genotyped on 64 SNPs in the ADH gene cluster. To enlarge the coverage of the region, 427 additional SNPs were imputed. Two different methods that correct for multiple SNP testing were used and compared: one method, based on linkage disequilibrium (LD), corrected for the effective number of SNPs tested; another method corrected for multiple testing by permutation.

Materials and Methods

Participants

Participants took part in a longitudinal survey on health, lifestyle and personality conducted in adult twins and their family members registered with the Netherlands Twin Register (NTR). Characteristics and recruitment of participants are described in Boomsma et al. (2002; 2006). Since 1991, every two to four years participants have received surveys with questions about health, lifestyle and personality. Twins were approached in 1991, 1993, 1995, 1997, 2000, 2002 and 2004/2005. Parents were invited to participate at all time points except 1997 and 2000. Siblings were asked to participate from 1995 onwards and spouses since 2000. Questions about alcohol use were included at all time points. In total, 23,173 individuals completed at least one questionnaire. For 1,774 individuals with information on alcohol phenotypes, genome-wide quality controlled SNP data were available as part of a genome-wide association study (GWAS) on Major Depressive Disorder (GAIN-MDD study). NTR participants in the MDD study were biologically unrelated, of Western European ancestry and mainly served as healthy controls. Detailed information on the GAIN-MDD study, recruitment and DNA collection can be found in Boomsma et al. (2008). Permission for the GAIN-MDD study was obtained from the Ethics Committee of Research on human subjects of the VU University Medical Center, Amsterdam.

Nearly all individuals with information on alcohol phenotypes had drunk alcohol at least once. Individuals who reported to have never drunk alcohol were excluded (n = 20; 1.1%). This resulted in a sample of 1,754 individuals (n = 1,112 female; 63.4%). The median year of birth was 1959 (ranging from 1923 to 1986). The majority of individuals (n = 1,204; 68.6%) provided data on three to seven surveys, 419 (23.9%) on two and 131 (7.5%) on one survey.

Phenotyping

For alcohol consumption variables, and problem drinking liability, reports were only included if the par-

ticipant was 26 or older at questionnaire completion. For age at onset variables and physical reactions to alcohol no restriction was set on the age of the respondent. The exact number of individuals with genotypic information differs per phenotype, not only because of age restrictions, but also because not all questions about alcohol use were included in all surveys.

Alcohol Consumption

Data on alcohol consumption were collected with two questions included at all time points except 1991. The first question was 'How often do you drink alcohol? Also count the times that you only drank a small amount (e.g. half a pint of beer or a few sips of wine)'. The response categories were *I don't drink alcohol, once a year or less, a few times a year, about once a month, a few times a month, once a week*, and *every day*. Based on this question four frequency measures were defined in 1,565 individuals:

- *Daily drinking (last* and *maximum reported)* analyzed as a dichotomous *yes/no* variable.
- *Weekly drinking or more (last* and *maximum reported)* analyzed as a dichotomous *yes/no* variable.

Last reported frequency of alcohol use measures was based on most recent information, for maximum reported frequency the measurement with the highest frequency was taken. For the 327 individuals who participated in only one survey, last reported and maximum reported frequency of alcohol consumption measures were based on the same measurement and thus similar.

The second question was 'How many glasses a week do you drink on average (including the weekend)?'. There were seven response categories: *less than 1 glass, 1–2 glasses a week, 3–5 glasses a week, 6–10 glasses a week, 11–20 glasses a week, 21–40 glasses a week* and *more than 40 glasses a week*. Based on this question two quantity measures were defined in 1,550 individuals:

- *Number of alcoholic drinks per week (last* and *maximum reported)* with six response categories (the categories *1–2 glasses a week* and *3–5 glasses* a week were combined).

For last reported number of drinks the most recent information was taken. Maximum number of drinks reported was based on the survey with the highest number of drinks reported. For the 353 individuals who participated in the survey only once, last reported and maximum number of drinks reported were based on the same measurement.

Age at Onset Alcohol Use

Information on age at onset of alcohol use was collected for three traits: experimental alcohol use (included in surveys in 1991, 1993, 1995, 1997 and 2000; n = 1,384), regular alcohol use (included in 1993, 1995, 1997 and 2000; n = 1,098) and getting drunk or tipsy (included in 1993, 1995 and 1997; n = 624). For these behaviors the question started with 'If you have ever used any of the substances listed below, please indicate the age at which you used them for the first time' and was followed by 'Tried an alcoholic drink', 'Regularly drank alcohol' and 'Gotten drunk or tipsy'. In 1991 age at onset was a continuous measure. In 1993, 1995 and 1997 there were eight response categories: *11 or younger, 12, 13, 14, 15, 16, 17* and *18 or older*. In 2000 response categories were brought back to five: *11 or younger, 12–13, 14–15, 16–17* and *18 or older*. Based on the three questions three phenotypes were defined:

- *Age at onset of experimental alcohol use* with the same response categories as in 2000.
- *Age at onset of regular alcohol use,* with the same categories as in 2000.
- *Age at onset of getting drunk* with response categories coded as *17 or younger* and *18 or older*.

Data were set to missing when no information was reported on age at onset. For individuals who provided data on age at onset of experimental alcohol use (n = 914), regular alcohol use (n = 592) or getting drunk (n = 278) on multiple surveys, the lowest reported age at onset was taken.

Physical Reactions to Alcohol

Information on experiencing unpleasant physical reactions after alcohol use was based on one question included in 1993 and 1995 (n = 872). This question 'Do you experience unpleasant physical reactions, like flushing of face or body, itching, sleepiness or heart beating after drinking one or two glasses of alcohol?' with response categories *always, sometimes* and *never* was used to define *Physical reactions to alcohol* with the analyzed categories *yes* (*always; sometimes*) and *no* (*never*). The 559 individuals who filled out this question in both years, were classified as experiencing reactions to alcohol if in one of the two surveys they had responded *yes*.

Problem Drinking Liability

Risk for problem drinking was assessed by the CAGE (Ewing, 1984) that was included at all time points except 1991. The CAGE consists of four items: 'Have you ever felt you should cut down on your drinking?', 'Have people annoyed you by criticizing your drinking?', 'Have you ever felt bad or guilty about your drinking?', 'Have you ever had a drink first thing in the morning to steady your nerves or to get rid of a hangover?'. Each item could be answered with *yes* or *no*. The number of yes items was used to define *Problem drinking liability* (n = 1,559). To take into account the possibility of nonrandom missing data and prevent assigning a low liability score to possible problem users, data of individuals were set to missing in case of two or more missing items. If one item was missing the mean value was imputed. For the 1,226

individuals who filled out the CAGE twice or more, the maximum score was taken.

Genotyping and Imputation

DNA was isolated from whole blood (Boomsma et al., 2008). Genotyping was performed by Perlegen Sciences using high-density oligonucleotide arrays. The 599,156 SNPs on these arrays had been selected to tag common variation in the Hapmap European and Asian panels (Sullivan et al., 2009). Genotyping procedures, genotyping calling rate algorithms and quality control procedures are available in the Supplementary Methods of Sullivan et al. (2009). In the ADH gene cluster on chromosome 4q successfully genotyped SNPs were selected (n = 64) ranging from base pair position 100186714 to base pair position 100626045 (build 36, release 24), to cover the same region as Macgregor et al. (2009).

SNPs that were not genotyped in this region, but that were included in the HapMap dataset were imputed. Imputation was done using Impute v0.5 (Marchini et al., 2007), with as a reference set the Hapmap2 CEU panel (build 36) which was obtained from the Impute website https://mathgen.stats.ox.ac.uk/impute/impute.html. Imputed genotypes were set to missing if none of the possible genotypes reached a maximum average genotype posterior probability of .70. Exclusion of SNPs with a minor allele frequency (MAF) < .01 (n = 152; all imputed) resulted in a final marker set of 491 SNPs of which 64 SNPs were genotyped and 427 imputed. Six of the 38 SNPs analyzed by Macgregor et al. (2009) were not included in the present study. Five of these were not genotyped as part of the GAIN-MDD study nor included in the HapMap2 dataset. One, the ADH1B polymorphism rs1229984, was imputed, but was excluded from further analyses since it had a MAF < .01.

Statistical Analyses

First, to examine the possible occurrence of selection bias, the genotyped sample was compared to a random sample of NTR participants without genotypic data, stratified by sex and age, on alcohol phenotypes. These comparisons were done in SPSS 15 by chi-squared tests on categorical variables and t-tests for independent samples on quantitative variables.

Next, associations between ADH polymorphisms and alcohol phenotypes were investigated by performing regression analyses in PLINK 1.06 (Purcell et al., 2007). Categorical data were analyzed with logistic regression; quantitative data were analyzed with linear regression. We modeled an additive effect and sex was included as covariate in each analysis. Age at completion of the survey was used as an additional covariate for last reported alcohol consumption measures. For maximum reported alcohol consumption variables, age at onset variables, problem drinking liability and reactions to alcohol we used year of birth as a covariate. SNPs in the ADH gene cluster were subdivided and analyzed within seven gene sets: ADH5, ADH4, ADH6, ADH1A, ADH1B, ADH1C and ADH7. These gene sets were defined as all SNPs ranging from the midpoint of one intergenic region until the midpoint of the next intergenic region, thereby including the SNPs in the particular gene. The outer gene sets spanned a larger intergenic region to include the same region as Macgregor et al. (2009): the ADH5 gene set was defined as all SNPs ranging from rs1230210 (in the gene METAP1, flanking the ADH region) to the midpoint of the intergenic region between ADH5 and ADH4; the ADH7 gene set was defined as all SNPs ranging from the midpoint of the intergenic region between ADH1C and ADH7 to rs1583971. The seven gene sets are described in Table 1.

Testing for associations between 491 genetic variants and 11 phenotypes posed the challenge how to deal with the problem of multiple testing. To reduce the type 1 error rate two methods to handle multiple SNP testing were applied and one method to handle multiple phenotype testing. The first method regarding multiple SNP testing was based on the correction of p values for the number of tests performed. Given the fact that the LD structure among SNPs is not independent, adjusting the p value for the actual number of tests would be overly stringent and result in a loss of power (Nyholt, 2004). With the first method the p values were therefore adjusted for the estimated number of *independent* SNPs tested. Calculation of

Table 1

Description of Gene Sets Within ADH Gene Cluster on Chromosome 4

Gene set	From SNP (bp[a])	To SNP (bp[a])	Total no. of SNPs	No. SNPs genotyped	No. SNPs imputed	M_{eff}[b]
ADH5	rs1230210 (100186714)	rs1840231 (100246202)	79	6	73	15.38
ADH4	rs6819724 (100247474)	rs10024022 (100307276)	119	11	108	15.88
ADH6	rs6532803 (100316196)	rs7439160 (100380677)	37	4	33	12.41
ADH1A	rs2173199 (100390402)	rs13145277 (100437458)	34	5	29	10.11
ADH1B	rs1229988 (100438024)	rs1789888 (100466593)	24	5	19	11.19
ADH1C	rs1789891 (100469442)	rs10516439 (100522181)	85	9	76	17.53
ADH7	rs2851292 (100522524)	rs1583971 (100626045)	113	24	89	30.34

Note: [a] Base pair position SNP as estimated in Hapmap2, build 36, release 24

[b] Effective number of SNPs in gene set (for explanation see paragraph on Statistical Analyses)

the number of independent SNPs (also called the effective number of SNPs; M_{eff}) was based on the number of eigenvalues of the n x n correlation matrix of allele frequencies of SNPs using equation 5 by Li and Ji (2005). For each gene set M_{eff} was estimated by an interface developed by Nyholt (2004), available at http://genepi.qimr.edu.au/general/daleN/SNPSpD/. SNPs in perfect LD ($r^2 = 1$) were automatically removed to obtain better estimates (Nyholt, 2005). With the estimates of M_{eff} as summarized in table 1, the asymptotic p value of the most significant SNP per gene set was adjusted for multiple testing by correcting the p value of the SNP for the estimate of M_{eff} of that gene set.

The second method to deal with the problem of testing multiple SNPs was based on permutation. In PLINK 1.06 (Purcell et al., 2007) set-based tests were performed using gene sets as described in table 1 to select up to n SNPs per gene set that were independently associated with the particular phenotype and derive an empirical p value based on permutation. This method had the advantage of selecting more than one significant SNP (if independent from each other) and could therefore give a better coverage of the association signal of the gene set than was possible with the first method employed. Set-based tests were performed as follows. First, within each gene set LD was calculated for each pair of SNPs. Next, regression analysis with sex and age (at completion or year of birth) as covariates was carried out. Based on these regression analyses, the most significant SNP per gene set with a threshold p value below .05 was selected (--set-p .05; default value). Other significant SNPs were selected ($p < .05$) in order of decreasing significance, only if these were relatively independent ($r^2 < .5$) from the SNPs already selected (--set-r2 0.5; default value) and if the number of selected SNPs did not exceed 5 (--set-max 5; default value). Since alcohol phenotypes can be seen as complex traits with many variants of small effects involved (O'Dushlaine et al., 2009), a threshold p value of .05 was chosen over a more stringent p value. Subsequently, for each gene set the mean of the test statistics of selected SNPs was calculated. Then, within the dataset, phenotype labels were permuted 10,000 times (--mperm 10000) keeping the LD between SNPs constant. For each permuted dataset SNPs were selected as described above (if $p < .05$; in order of decreasing significance; independent of already selected SNPs) and for each permutation set the mean of test statistics of selected SNPs was calculated. This resulted in a distribution of mean test statistics that was used to calculate an empirical p value for each gene set and phenotype based on the number of mean test statistics from the permuted datasets that exceeded the original mean test statistic of the actual dataset.

Lastly, the fact that multiple phenotypes were tested ($n = 11$) was accounted for. This correction was done in addition to the corrections for multiple SNP testing described above. Since the phenotypes tested were correlated, applying a Bonferroni correction by dividing the significance level of .05 by the actual number of phenotypes ($n = 11$), would be too conservative and would result in a loss of power (Nyholt, 2004). Therefore correction for multiple phenotype testing was done by correcting the significance level of .05 for the number of *independent* phenotypes. An estimate of the number of independent phenotypes, derived from the number of eigenvalues of the n x n correlation matrix of phenotypes, was based on equation 5 given by Li and Ji (2005), analogous to the estimation of M_{eff}, and obtained by another interface developed by Nyholt (2004), available at http://gump.qimr.edu.au/ general/daleN/matSpD/. The number of independent phenotypes in this study was estimated to be 7. This resulted in a significance level of .05/ 7 = .007. Therefore, for all analyses conducted, a p value of .007 or less was considered significant.

Results

Comparison of Genotyped Sample With Random Sample

Table 2 shows that individuals in the genotyped sample were comparable to individuals in the random NTR sample in last reported alcohol consumption measures, age at onset variables, problem drinking liability and in the frequency of experiencing physical reactions to alcohol. Based on maximum reported alcohol consumption measures however, individuals in the genotyped sample more often reported to have drunk alcohol daily and weekly and reported to have drunk more glasses of alcohol per week than individuals in the random NTR sample (p values < .007).

Associations of ADH Polymorphisms With Alcohol Phenotypes

For each phenotype Tables 3–6 show the unadjusted asymptotic p values, the p values adjusted for the number of independent SNPs per gene set and the empirical p values for the most significant SNPs per gene set.

Alcohol Consumption

None of the ADH polymorphisms were significantly associated with alcohol consumption measures after correction for multiple SNP and phenotype testing, for either of the test methods applied (see Table 3).

Age at Onset of Alcohol Use

Based on empirical p values derived by permutation, but not on adjusted p values (corrected for the number of independent SNPs), one polymorphism in the ADH7 gene set (rs2654849), located 40.4 kb upstream of the ADH7 gene (base pair position 100615713, build 36) was significantly associated with age at onset of regular alcohol use (empirical p value = .003), as can be seen in Table 4. Individuals who started regular alcohol use at an earlier age more often have the minor G allele at this locus ($\beta = -.10$;

Table 2

Description of Alcohol-Related Phenotypes for Genotype Sample and Comparison with Random Stratified Sample

		Genotype sample	Comparison sample	Test statistic
Ever used alcohol	*N*	1774	1774	$\chi^2(1) = .212$, $p = .645$
	Yes (%)	1754 (98.9)	1751 (98.7)	
Daily drinking (last reported)	*N*	1565	1750	$\chi^2(1) = 3.235$, $p = .072$
	Yes (%)	324 (20.7)	319 (18.2)	
Daily drinking (maximum reported)	*N*	1565	1750	$\chi^2(1) = 14.306$, $p < .001$*
	Yes (%)	404 (25.8)	355 (20.3)	
Weekly drinking or more (last reported)	*N*	1565	1750	$\chi^2(1) = 4.281$, $p = .039$
	Yes (%)	1028 (65.7)	1089 (62.2)	
Weekly drinking or more (maximum reported)	*N*	1565	1750	$\chi^2(1) = 15.830$, $p < .001$*
	Yes (%)	1128 (72.1)	1149 (65.7)	
No. drinks per week (last reported)	*N*	1550	1736	$\chi^2(5) = 9.367$, $p = .095$
	< 1 (%)	454 (29.3)	540 (31.1)	
	1–5 (%)	491 (31.7)	606 (34.9)	
	6–10 (%)	281 (18.1)	280 (16.1)	
	11–20 (%)	252 (16.3)	243 (14.0)	
	21–40 (%)	65 (4.2)	61 (3.5)	
	> 40 (%)	7 (.5)	6 (.3)	
No. of drinks per week (maximum reported)	*N*	1550	1736	$\chi^2(5) = 25.746$, $p < .001$*
	< 1 (%)	359 (23.2)	485 (27.9)	
	1–5 (%)	484 (31.2)	591 (34.0)	
	6–10 (%)	298 (19.2)	307 (17.7)	
	11–20 (%)	295 (19.0)	270 (15.6)	
	21–40 (%)	103 (6.6)	72 (4.1)	
	> 40 (%)	11 (0.7)	11 (.6)	
Age at onset experimental use	*N*	1384	905	$\chi^2(4) = 13.526$, $p = .009$
	< 12 (%)	104 (7.5)	38 (4.2)	
	12–13 (%)	177 (12.8)	101 (11.2)	
	14–15 (%)	423 (30.6)	277 (30.6)	
	16–17 (%)	422 (30.5)	300 (33.1)	
	18+	258 (18.6)	189 (20.9)	
Age at onset regular use	*N*	1098	698	$\chi^2(4) = 4.268$, $p = .371$
	< 12 (%)	3 (0.3)	5 (.7)	
	12–13 (%)	12 (1.1)	7 (1.0)	
	14–15 (%)	103 (9.4)	55 (7.9)	
	16–17 (%)	311 (28.3)	184 (26.4)	
	18+ (%)	669 (60.9)	447 (64.0)	
Age at onset getting drunk	*N*	624	351	$\chi^2(1) = 4.353$, $p = .037$
	< 18 (%)	239 (38.3)	111 (31.6)	
Physical reactions to alcohol	*N*	872	512	$\chi^2(1) = .890$, $p = .346$
	Yes (%)	225 (25.8)	144 (28.1)	
Problem drinking liability	*N*	1559	1748	$t(3305) = -.584$, $p = .559$
	M	.476	.459	
	SD	.832	.855	

Note: * = significant at the $\alpha = .007$ level (adjusted for multiple phenotype testing)

MAF = .34; major allele = T). Other associations between ADH polymorphisms and age at onset measures were not detected.

Physical Reactions to Alcohol

Table 5 shows that a noncoding genetic variant in ADH5, rs6827292, was significantly associated with experiencing unpleasant physical reactions to alcohol, based on its empirical *p* value ($p = .001$). Each copy of a C-allele (MAF = .044) conveyed an increased risk for experiencing physical reactions to alcohol, compared to carrying a T-allele (odds ratio (OR) = 2.426; base pair position 100212017, build 36). Its *p* value adjusted for the effective number of SNPs tested, was marginally significant (adjusted *p* value = .008). The same applied for the genetic variant rs6819724 in the ADH4 gene set. Based on its empirical *p* value the marker rs6819724 was significantly associated with experiencing physical reactions to alcohol (empirical *p* value = .0007), but considering its adjusted *p* value, rs6819724 was only marginally significant (adjusted *p* value = .008). Each copy of a G-allele at this locus (MAF = .045) increased the risk of experiencing physical reactions, compared to carrying an A-allele (OR = 2.417). Rs6819724 was located 18.7 kb upstream of

Table 3

Most Significant SNPs Per Gene Set (Based on Asymptotic *p* Values Adjusted for the Effective no. of SNPs and Empirical *p* Values) for Measures of Alcohol Consumption

	Drinking daily (n = 1,565)								Drinking weekly or more (n = 1,565)								No. of alcoholic drinks per week (n = 1550)							
	Last reported				Maximum reported				Last reported				Maximum reported				Last reported				Maximum reported			
Gene set	SNPs	Min. p[b]	Adj. p[c]	Emp. p[d]	SNPs	Min. p[b]	Adj. p[c]	Emp. p[d]	SNPs	Min. p[b]	Adj. p[c]	Emp. p[d]	SNPs	Min. p[b]	Adj. p[c]	Emp. p[d]	SNPs	Min. p[b]	Adj. p[c]	Emp. p[d]	SNPs	Min. p[b]	Adj. p[c]	Emp. p[d]
ADH5	rs1061187	.016	.246	.155	rs1230201	.079	1	1[f]	rs17216887	.024	.369	.369	rs1230205	.027	.415	.304	rs17216887	.036	.554	.432	rs1312200	.024	.369	.389
									rs17216446[e]								rs1061187[e]				rs1230201[e]			
									rs1230201[e]								rs1230201[e]							
									rs2924584[e]								rs6827292[e]							
ADH4	rs17218003	.061	.969	1[f]	rs7694844	.073	1	1[f]	rs7694844	.070	1	1[f]	rs7694844	.088	1	1[f]	rs10026860	.032	.508	.382	rs7694844	.015	.238	.132
																	rs6819724[e]							
ADH6	rs7661441	.160	1	1[f]	rs4699734[g]	.060	.745	1[f]	rs4699734[g]	.123	1	1[f]	rs6532803[i]	.088	1	1[f]	rs4699734[g]	.014	.174	.098	rs4699734[g]	.034	.422	.274
ADH1A	rs904092	.148	1	1[f]	rs12506882	.053	.536	1[f]	rs904092	.076	.768	1[f]	rs904092	.009	.091	.059	rs12506882	.015	.152	.133	rs12506882	.036	.364	.281
																	rs904092[e]							
ADH1B	rs1789882	.191	1	1[f]	rs17033	.058	.649	1[f]	rs1789882	.076	.850	1[f]	rs1789882	.015	.168	.120	rs3811802	.022	.246	.218	rs17033	.066	.739	1[f]
																	rs1789882[e]							
																	rs17033[e]							
ADH1C	rs1826907	.036	.631	.456	rs1826907	.052	.912	1[f]	rs3114046[h]	.205	1	1[f]	rs1814125	.129	1	1[f]	rs1229864	.138	1	1[f]	rs283410	.142	1	1[f]
ADH7	rs12505135	.017	.516	.475	rs12505135	.010	.303	.369	rs284787[i]	.031	.941	.583	rs11933667	.031	.941	.552	rs1154486	.034	1	.628	rs3805331	.094	1	1[f]
	rs7667212[e]				rs1372680[e]				rs894369[e]															

Note: [b] Asymptotic *p* value of most significant SNP in gene set, unadjusted for multiple SNP testing.

[c] Asymptotic *p* value of most significant SNP in gene set adjusted for the effective number of SNPs in gene set = Min. $p * M_{eff}$ (from Table 1).

[d] Empirical *p* value of selected SNPs (most significant SNP if asymptotic *p* value < .05 and, if applicable, other SNPs that are independent, $r^2 < .5$) of SNPs already selected).

[e] Additional SNP selected by set-based test (having a *p* value < .05 and being independent of SNP(s) already selected).

[f] No SNPs were selected by set-based test (*p* value most significant SNP > .05), therefore empirical *p* value could not be calculated.

[g] SNP rs4699734 is in LD 1 with SNPs rs7375429; these SNPs have the same *p* value.

[h] SNP rs3114046 is in LD 1 with SNPs rs1391088; these SNPs have the same *p* value.

[i] SNP rs284787 is in LD 1 with SNPs rs284784 and rs1827567; these SNPs have the same *p* value.

[j] SNP rs6532803 is in LD 1 with SNPs rs2213039; these SNPs have the same *p* value.

* = significant at the $\alpha = .007$ level (adjusted for multiple SNP and phenotype testing).

Table 4

Most Significant SNPs Per Gene Set (Based on Asymptotic *p* Values Adjusted for the Effective Number of SNPs and Empirical *p* Values) for Measures of Age at Onset of Alcohol Use

	Experimental alcohol use (*n* = 1,384)				Regular alcohol use (*n* = 1,098)				Getting drunk (*n* = 624)			
Gene set	SNPs	Min. p^b	Adj. p^c	Emp. p^d	SNPs	Min. p^b	Adj. p^c	Emp. p^d	SNPs	Min. p^b	Adj. p^c	Emp. p^d
ADH5	rs17028457	.014	.215	.139	rs1230201	.134	1	1[f]	rs10084896	.074	1	1[f]
ADH4	rs6837685[k]	.154	1	1[f]	rs1540053	.146	1	1[f]	rs2156731	.097	1	1[f]
ADH6	rs4699734[g]	.198	1	1[f]	rs2097122	.137	1	1[f]	rs12507078	.149	1	1[f]
ADH1A	rs17028765	.239	1	1[f]	rs1230024	.241	1	1[f]	rs12506882	.206	1	1[f]
ADH1B	rs1229982	.107	1	1[f]	rs4147536	.194	1	1[f]	rs17033	.126	1	1[f]
ADH1C	rs1826907	.259	1	1[f]	rs7661978	.063	1	1[f]	rs4699743	.058	1	1[f]
ADH7	rs7663410	.004	.121	.031	rs2654849	.0007	.021	.003*	rs1583971	.056	1	1[f]
	rs2584448[e]											

Note: [k] SNP rs6837685 is in LD 1 with SNPs rs4699710; these SNPs have the same *p* value.
For further legend, see Table 3.

the ADH5 gene (base pair position 100247474, build 36). Additional analyses showed that rs6827292 and rs6819724 were in LD 1 with each other. SNP rs6819724, located just intermediate of the genes ADH4 and ADH5, was included in gene set ADH4. Therefore a significant result was obtained in both gene sets.

Problem Drinking Liability

Table 6 summarizes the test results for the associations between ADH polymorphisms and problem drinking liability per gene set. None of the *p* values reached significance after correction for multiple SNP and phenotype testing by either method.

Discussion

The aim of the present study was to replicate the associations between polymorphisms in the ADH gene cluster and alcohol consumption variables and physical reactions to alcohol previously demonstrated by Macgregor et al. (2009). Associations between ADH polymorphisms and age at onset of alcohol use and problem drinking liability were also studied. The associations between genetic variants in the ADH1B, ADH1C, ADH4 and ADH5 genes and current alcohol use which were found by Macgregor et al. (2009) were not replicated in the current study. Because of the low MAF of the ADH1B polymorphism rs1229984, the associations of this SNP with alcohol phenotypes could not be tested. Our study does however show that three other polymorphisms in the ADH gene cluster are associated with alcohol phenotypes. The genetic marker rs2654849, located 40.4 kb upstream of ADH7, was found to be associated with age at onset of regular alcohol use. Two other SNPs in high LD with each other were associated with experiencing physical reactions to alcohol: rs6827292, a noncoding genetic variant located in ADH5 and rs6819724 located 18.7 kb upstream of ADH5. At this point it is unknown whether one of these markers is a causal variant itself, or whether they are in LD with the causal variant. Interestingly, Edenberg et al. (2006)

Table 5

Most Significant SNPs Per Gene Set (Based on Asymptotic *p* Values Adjusted for the Effective Number of SNPs and Empirical *p* Values) for Physical Reactions to Alcohol (*n* = 872)

Gene set	SNPs	Min. p^b	Adj. p^c	Emp. p^d
ADH5	rs6827292	.0005	.008	.001*
ADH4	rs6819724	.0005	.008	.0007*
ADH6	rs7439160	.053	.658	1[f]
ADH1A	rs17028770	.025	.253	.182
ADH1B	rs1693439	.039	.436	.332
ADH1C	rs17586246	.038	.666	.449
ADH7	rs2718682	.003	.091	.151
	rs11933667[e]			
	rs7696921[e]			

Note: For legend, see Table 3.

Table 6

Most Significant SNPs Per Gene Set (Based on Asymptotic *p* Values Adjusted for the Effective Number of SNPs and Empirical *p* Values) for Problem Drinking Liability (*n* = 1,558)

Gene set	SNPs	Min. p^b	Adj. p^c	Emp. p^d
ADH5	rs1154400[k]	.054	.831	1[f]
ADH4	rs2851247	.053	.842	1[f]
ADH6	rs6830685	.028	.347	.224
ADH1A	rs904092	.022	.222	.169
ADH1B	rs1693457	.021	.235	.202
	rs6810842[e]			
ADH1C	rs2866152	.067	1	1[f]
ADH7	rs12505135	.019	.576	.330

Note: [k] SNP rs1154400 is in LD 1 with SNPs rs1311620; these SNPs have the same *p* value.
For further legend, see Table 3.

reported significant SNPs in the same region between ADH4 and ADH5 for alcohol dependence. The specific significant variants detected in this study have not been reported in the literature before for the alcohol phenotypes that we studied or related phenotypes. Still, the number of studies focusing on variants across the entire ADH region is small. So far, only four studies have been conducted in populations from Western European descent (Edenberg et al., 2006; Kuo et al., 2008; Luo et al., 2006; Macgregor et al., 2009).

Physical reactions to alcohol were linked to SNPs in different genetic regions than in previous studies. The 'Asian flush' has been related to variants in the ALDH2 gene on chromosome 12 and, to a lesser extent, in the ADH1B gene that we also studied (Chen et al., 1998; Shibuya et al., 1989; Takeshita et al., 1996). Variants in these genes are hypothesized to change the rate at which alcohol is metabolized. The resulting excess of acetaldehyde is considered to give heightened reactions to alcohol (Eng et al., 2007). In the present study, reactions to alcohol were associated with the ADH5 gene polymorphism rs6827292 and the nearby SNP rs6819724. This raises the question whether reactions to alcohol reported in this study are comparable to the Asian flushing response characterized by facial flushing, nausea, and tachycardia (Brooks et al., 2009). In line with the description of the Asian flush given by Brooks et al. (2009) and Chen et al. (1998), the present study inquired whether people experienced reactions as flushing of face or body, itching, sleepiness or heart beating after drinking one or two glasses of alcohol. Experiencing nausea was not included in the question however. Further research is necessary to investigate which regions of the ADH gene cluster are related to experiencing physical reactions to alcohol in Western populations and by what mechanism.

The genetic variant rs2654849 was associated with age at onset of regular alcohol use. Large studies in adults have demonstrated that age at onset of drinking is associated with alcohol dependence, with people that started drinking early being more likely to experience (symptoms of) alcohol dependence (Hingson et al., 2006; Dawson et al., 2008). Furthermore, twin studies have shown that age at onset of alcohol use and alcohol dependence status are correlated at the genetic level with a genetic correlation of .54–.59 indicating substantial overlap in the genetic factors that affect both traits (Grant et al., 2006; Sartor et al., 2009). In future studies it would therefore be interesting to test whether the SNP rs2654849 is also associated with alcohol dependence.

Variants of ADH genes are assumed to encode enzymes that influence the rate by which ethanol is metabolized (Edenberg, 2007). Birley et al. (2009) investigated which genetic variants in the ADH gene cluster were associated with alcohol metabolism in vivo. They found inter-individual variation in the early stages of alcohol metabolism (absorption of ingested alcohol) to be associated with SNPs in or near ADH7 and also with SNPs in the ADH1A, ADH1B, ADH4 genes. Variance in the elimination rate of alcohol was related to markers in the ADH1B and ADH1C genes and to variants located in the intergenic region between ADH1C and ADH7. About 20% of the genetic variance in alcohol metabolism could be explained by the combined effects of these SNPs (Birley et al., 2009). These findings were unlinked to the significant SNPs in the present study. This could indicate that the ADH gene cluster is also related to alcohol use by other mechanisms than the metabolizing pathway. Examples of other pathways that can relate genes to alcohol traits are sensitivity to the effects of alcohol (level of response) or personality traits that increase the risk of substance related problems such as impulsivity, sensation seeking and disinhibition (Schuckit, 2009).

Further evidence for the role of the ADH gene cluster in explaining individual differences in alcohol use comes from studies among Western populations of European descent that have looked at alcohol dependency. These studies demonstrated positive associations with alcohol dependence for ADH1A markers (Edenberg et al., 2006), haplotypes (Kuo et al., 2008) and diplotypes (Luo et al., 2006). A meta-analysis showed variants in the ADH1B to be associated with alcoholism (Zintzaras et al., 2006). They did not find evidence for an association between variants in the ADH1C gene and alcoholism, although a recent study did detect haplotypic association between the ADH1C gene and alcohol dependence (Kuo et al., 2008). This discrepancy might be explained by the fact that variants in the ADH1B and ADH1C genes are in strong LD, the risk for alcoholism associated with the ADH1C gene possibly being attributable to a variant in the ADH1B gene (Chen et al., 1999; Osier et al., 1999). Positive associations between alcohol dependence and ADH gene variation have also been detected for ADH4 genotypes (Luo et al., 2006) and haplotypes (Edenberg et al., 2006), for ADH5 genotypes (Luo et al., 2006) and haplotypes (Kuo et al., 2008) and variants in the ADH7 gene (Edenberg et al., 2006). Whether these variants are specific to alcohol dependency or also influence alcohol use, awaits further study.

Testing 491 ADH polymorphisms provided a detailed picture of the association signal in the ADH region. Yet it had the drawback of increasing the multiple testing problem. This was dealt with in two ways. The first method corrected for multiple testing by adjusting the asymptotic p value of the most significant SNP in a gene set for the effective number of SNPs in that set. The second method was based on empirical p values derived by permutation. Both adjusted and empirical p values were subjected to a significance-level corrected for testing of multiple phenotypes as well. When comparing the performance of the two methods to correct for multiple testing,

adjusting *p* values for the effective number of SNPs seems a slightly more conservative approach than correction based on permutation. Three SNPs were significant considering their empirical *p* values, but not (rs2654849) or only marginally significant (rs6827292 and rs6819724) when considering the *p* values adjusted for the effective number of SNPs in the gene set. This difference in *p* values might be due to an overestimation of M_{eff} resulting from the fact that higher order SNP correlations are not captured in the pairwise LD estimates on which M_{eff} is based (Nyholt, 2005). In line with the observed differences in *p* values in this study, the literature considers using empirical *p* values based on permutation one of the best ways to correct for multiple testing (Knight et al., 2008).

A possible limitation of the current study is that most individuals in the genotyped sample were selected as having a low liability for MDD to serve as control in the GAIN-MDD study. This might have caused a difference in the comparability between our sample of hyper controls and the Australian sample. The comparison between the genotyped NTR sample and random NTR sample however shows few differences for alcohol phenotypes, in spite of reported associations between alcohol consumption and depression (Graham et al., 2007). Individuals in the genotyped sample more often reported to have drunk alcohol daily and weekly and reported higher quantities of alcohol use, but not more problem drinking than in the random NTR sample. This finding is in line with the existing research that moderate drinking is associated with less mental health problems compared to light or heavier drinking (Chen et al., 2006). The comparability of this sample to the Australian sample is further strengthened by the agreement in frequencies of alcohol phenotypes, such as alcohol use ever and alcohol use in the previous 12 months.

A further limitation is that the sample size of the replication study was smaller than that of the Australian discovery sample. Obviously, this influenced the power to detect effects of the size found by Macgregor et al. (2009). We carried out ad-hoc power analyses for phenotypes with different prevalence. For phenotypes for which the prevalence was low, the power to detect significant effects after correcting for multiple SNP testing (within gene sets) and multiple phenotype testing was low regardless of relative risk (RR). For phenotypes with a higher prevalence (around 65%; e.g. weekly drinking) power was still relatively low for a RR of 1.1 (between .25 and .55 for SNPs with a MAF ranging from .20 to .40). For a RR of 1.2 or higher the power was very good (> .95). Thus, for most phenotypes we had sufficient power to detect associations with modest to large effect sizes (RR > 1.2), but our power to detect very small effect sizes was low (RR ~ 1.1).

In order to maximize coverage of the genetic variation in the ADH gene cluster, we imputed all SNPs in HapMap2 that were not genotyped. This is a sensible route to undertake given that the LD structure in the present study is similar on a genome-wide level to the HapMap reference set (Pardo et al., 2009). Still, six genetic markers could not be analyzed. The top SNP in Macgregor et al. (2009), rs1229984, was imputed, but had to be discarded from the analyses, since its MAF was < .01 (based on the HapMap2-CEU sample). Five other markers analyzed by Macgregor et al. (2009) could not be imputed, as they were not included in the HapMap2-CEU reference set. A total of 491 SNPs was measured or imputed across the ADH region. In our analyses we divided the region into seven gene sets, which corresponded with the known genes in this region. Corrections for multiple testing were carried out within gene sets. It could be argued that the entire ADH region should be considered as one gene set. In that case the association between age at onset of regular alcohol use and SNP rs2654849 is still significant (empirical *p* value = .004). The associations of physical reactions with rs6827292 and rs6819724, for which data on 872 individuals were available, would however no longer be significant (empirical *p* value = .127).

In conclusion, the present study contributes to the existing evidence that the ADH gene cluster is important for understanding the genetics of alcohol use.

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